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USE OF AN ENZYME FOR THE MANUFACTURE OF AN AGENT FOR CONTROLLING BACTERIAL INFECTION

The present invention is directed to the use of an enzyme for the manufacture of an agent for the treatment and/or prophylaxis of a bacterial infection.

The farming of many different types of animals is important throughout the world for the production of food for human consumption. When the animals are reared, they come into contact with a variety of infection-causing bacteria, such as *Campylobacter* and *Salmonella*. In some cases these bacteria may spread directly from animals to humans (zoonosis). Accordingly, it is necessary from an economic, environmental and health perspective that such bacterial infection is prevented or eradicated in the animal prior to human consumption to prevent the spread of the disease to humans.

The domestic animal of particular, but not exclusive, concern with regard to zoonosis is the chicken. *Campylobacter* and *Salmonella* are particularly prevalent in the chicken. The bacteria are transmitted to the bird in a variety of ways, including through feed, water, litter and vermin. The bacteria initially infect the caecae of the chicken. The disease then progresses to the small intestine where infestation may cause loss of weight in the bird. A particular problem with the chicken

is that it is almost impossible to eviscerate in a sterile manner with the result that bacteria inhabiting the intestines will invariably be transmitted to the saleable carcass. Accordingly the potential for zoonosis is great, unless the carcass is handled or cooked properly. The cost of human infection caused by eating improperly treated chicken is significant in terms of both time and lives.

Accordingly, presently there is a demand for improved methods of reducing bacterial infection in animals such as the chicken, in particular those intended for human consumption.

Various solutions to the problem of bacterial infection have been proposed. Current methods of control include the application of antibiotics, feed sterilisation and careful and controlled handling and cooking of the carcass after slaughter. Feed sterilisation has proved ineffective in the absence of a sterile rearing environment (which is impractical) whilst controlled handling and cooking cannot be relied upon in every instance. The application of antibiotics has proved unpopular with consumer groups wishing to reduce the quantity of potentially harmful chemicals in food. The use of antibiotics has the additional problem that if they are not introduced into the animal in a properly controlled manner, antibiotic-resistant strains of bacteria can be created, making such infections more difficult to treat in the future. The prophylactic use of

antibiotics in animal feed has thus been regulated in some countries (notably Sweden and Finland) effectively reducing the available methods of control. Indeed no single method provides a barrier which completely prevents bacteria being transferred from the animal to humans.

As an alternative to the above methods it has been proposed in *Poultry Science, 1994* 73:402-407, to introduce flora into chickens to compete with the bacteria causing the infection. Such mucosal competitive exclusion flora (MCE) were found to reduce the level of *Campylobacter jejuni* infection in chickens. However, the competitive exclusion treatment is not found to be consistently effective, its efficacy varying from animal to animal.

JP-A-81-73055 discloses animal feeds intended to prevent contamination with *Salmonella*. The feeds are indicated to contain partially decomposed mannan in the form of mannose polysaccharides. These are produced by degradation of mannan with an enzyme, produced by micro-organisms. The resulting feed was found to be moderately effective against *Salmonella* in chickens, but is not effective against *Campylobacter*.

US-A-5 124 262 discloses a mannose isomerase enzyme used for converting fructose to mannose. The mannose thus produced is taught to be useful in feeds, for inhibiting the growth of *Salmonella* in chickens.

In Bamboo J. 1993, pp. 29-35, xylan prepared from steamed bamboo grass is mentioned as inhibiting the growth of various human intestinal bacteria. In particular the xylan is indicated to be effective against *Salmonella*. However, the inhibition effect is reversed after a period of 24 hours.

The above methods have proved more desirable from an environmental and health point of view, than the administration of antibiotics. However, none have proved effective enough to be commercially viable.

WO 93/01800 discloses the use of a protease for the preparation of a medicament effective against intestinal pathogens in animals. The pathogens of interest include *Campylobacter*. However, there is no mention of enzymes other than proteases being useful in controlling animal pathogens.

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EP-A-0 681 787 discloses use of a carbohydrase or protease for the manufacture of an agent for the treatment of *Coccidiosis*. However, *Coccidiosis* in chickens is caused by protozoal oocytes. The document does not indicate how bacterial pathogens in chickens, or other animals, can be controlled.

Accordingly, one object of the present invention is to provide an agent which can be used for controlling bacterial infection that is more effective than the

presently available agents, and in particular than those described in the prior art acknowledged above. A further object of the present invention is to provide an agent which can be used for controlling bacterial infection that is less harmful to the environment, less expensive than the presently available agents, and has advantages for human health.

Accordingly, the present invention provides the use of a xylanase or a cellulase for the manufacture of an agent for the treatment and/or prophylaxis of bacterial infection in an animal caused by *Salmonella*, *Campylobacter* or *Clostridium perfringens*.

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A preferred cellulase is β-glucanase.

Figure 1 shows the effect of three diets on *Campylobacter* colonisation in 12-day old chicks. Three different dilution levels of the initial stock solution of *Campylobacter* were used to introduce the pathogen to the chicks. The results are presented as mean scores of positive caecae and represent the combined results of two flocks A and B comprising a total of 108 birds (12 per dilution for each of the three diets).

Figure 2 shows the results of Figure 1, but for flock A alone.

Figure 3 shows the results of Figure 1, but for flock B alone.

Figure 4 shows similar results to Figure 1, but at three alternative dilution levels of the initial *Campylobacter* stock solution. The results are presented as mean counts $\log_{10}$CFU(Colony Forming Units)/ml.

Figure 5 shows the results of Figure 4, but for flock A alone.

Figure 6 shows the results of Figure 4, but for flock B alone.

Figure 7 shows the effect of three diets on *Campylobacter* colonisation of the small intestine and caecae of 17-day old chicks. The results are presented as mean counts $\log_{10}$CFU/ml, and represent the combined results of two flocks A and B comprising a total of 72 birds (12 per treatment) for each of the three diets.

Figure 8 shows the results of Figure 7, but for flock A alone.

Figure 9 shows the results of Figure 7, but for flock B alone.

Figure 10 shows a comparison of the weight of 1, 5, 12, 19, 25 and 33-day old chicks (20 in total) dosed with *Campylobacter jejuni*, and similar chicks (25 in total) which have not been dosed, the chicks all being fed a wheat-based diet.

Figure 11 shows similar results to those of Figure 10, but for chicks on a wheat plus xylanase diet.

Figure 12 shows similar results to those of Figure 10, but for chicks on a maize-based diet.

Figure 13 shows the effect of two different diets (wheat and wheat plus xylanase) on *Salmonella enteritidis* colonisation in 14 day old chicks. The results are presented as mean counts $\log_{10}$CFU/ml. Tests were carried out on two flocks, A and B, comprising a total of 48 birds, 24 per diet.

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The advantage of using feeds containing a xylanase or a cellulase for rearing animals is that the amount of antimicrobial drugs which have previously been routinely incorporated into their diet can be reduced, or in some cases omitted entirely. This enables considerable economic savings to be achieved in view of the relative expense of antibiotics. In countries where such drugs are banned, it represents a totally new approach to the control of bacterial diseases.

When omitting antibiotics from an animal's diet there are several potential further benefits. It has previously been necessary to withdraw antibiotics from the animal's diet for a certain time prior to slaughter. This ensures that the meat is relatively free from such drugs and thus fit for human consumption. In contrast, if antibiotics

are entirely omitted from an animal's diet, as may be achieved with the present invention, then the animal can be slaughtered at any age rather than after a certain withdrawal period. This affords the farmer improved flexibility and removes the risk of animals becoming infected shortly prior to slaughter. Further, meat and eggs can be guaranteed free of antibiotics. Such meat and eggs have a market advantage as compared to products which cannot support such a guarantee.

Even if the enzyme added to the animal's diet only enables the level of inclusion of antibiotics to be reduced, then the overall cost of controlling bacterial infection will be reduced. Synergy or potentiation may extend the useful life of the antibiotic.

The present invention also has benefits for human health. Its use reduces the selection pressure for antibiotic-resistant strains of bacteria, by allowing antibiotics to be removed from animal feed. Accordingly, more antibiotic-susceptible strains will be present in the gut of the animal, thereby ensuring a more likely positive outcome in the event of antibiotics being used on the equivalent human condition.

The xylanase or cellulase enzyme to be used in the feeds can be formulated as a pre-mix together with any other enzymes to be included. The pre-mix can be added to the raw materials before feed manufacture, during feed manufacture or as a final step once the feed is otherwise

ready for use. It is possible to add the enzyme directly as a liquid to a feed material pre-formed as pellets or as a mash.

It is also possible to include the enzyme in the animal's diet by incorporating it into a second (and different) feed or drinking water which the animal also has access to. Accordingly, it is not essential that the enzyme is incorporated into the feed itself, although such incorporation forms a particularly preferred aspect of the present invention.

If the enzyme is incorporated into a feed, then this preferably comprises at least 25% by weight of a cereal, and more preferably at least 35% by weight of the cereal. The cereal may be any one or more of wheat, maize, rye, barley, oats, triticale, rice, and sorghum. It is particularly preferred that the cereal is wheat.

Although the cereal component of a cereal-based diet constitutes a source of protein, it is usually necessary to include sources of supplementary protein in the diet, such as those derived from fishmeal, meatmeal or vegetables. These sources of supplementary protein may constitute up to 50% by weight of the animal feed. Sources of vegetable protein include at least one of full fat soybean, rapeseed, canola, soybean meal, rapeseed meal and canola meal.

If the enzyme is incorporated into a feed, then this is preferably added in a relative amount of 0.0001-10 g of the enzyme per kilo of the feed, more preferably 0.001-1 g/kg and most preferably 0.01-0.1 g/kg.

The xylanase for use in this invention can be obtained from a fungus, such as *Trichoderma*, *Aspergillus*, *Humicola*, or *Neocallimastix*. Alternatively, the xylanase can be obtained from a bacterium, such as *Bacillus*, *Streptomyces*, *Clostridium*, or *Ruminococcus*.

The present invention is particularly effective against strains of *Salmonella* and *Campylobacter*, and especially *Salmonella enteritidis* and *Campylobacter jejuni*. Another bacterium against which the invention is effective is *Clostridium perfringens*.

Bacterial infection can be treated or prevented in accordance with the present invention in a wide variety of animals, but use of the invention is particularly preferred in domestic animals and farm livestock. Animals which may in particular benefit from the invention include poultry (such as chickens, turkeys, ducks and geese), ruminants (such as cattle, horses and sheep), swine (pigs), cats, dogs, rodents (such as rabbits) and fish. The invention is particularly useful in broiler chickens.

The most preferred combinations of feed and enzyme include wheat plus xylanase, maize plus xylanase and barley plus β-glucanase.

The enzymes used in the present invention fall within a general Class called polysaccharidases. Their substrates are structural polysaccharides such as xylans and β-glucans that occur as an integral part of the cell wall of most land plants. These polysaccharides are not found in animal cells, and are not to be expected to have any activity against proteins. Because these enzymes attack polysaccharides found in plant cell walls, the only possible substrates for these enzymes in the gastrointestinal tract of an animal are contained in cereal-based feeds. It is therefore speculated that the beneficial effects of the xylanase or cellulase on bacterial infection result somehow from the degradation products which they produce such as xylan or β-glucan derived from a cereal-based diet.

As previously mentioned, WO 93/01800 discloses the use of a protease for the preparation of a medicament effective against intestinal pathogens in animals. It is well established that such pathogens mediate their infectivity by binding to receptors on the surface of intestinal epithelial cells via antigenic protein or glycoprotein molecules expressed on the pathogen's cell surface. It is suggested that the protease enzyme prevents binding of the pathogen cells to the intestinal epithelium by destroying these proteinaceous receptor/adhesion sites to

which the pathogen must bind if it is to cause an infection. The protease enzymes mentioned in this reference would be predicted to destroy proteins on the lumenal surface of intestinal epithelial cells in a non-specific manner, but would not be expected to attack substrates other than proteins. Accordingly, the activity of the proteases disclosed in this reference is fundamentally different from the activity of the enzymes used in the present invention. A skilled person could not have predicted the utility of the present enzymes against bacterial infection based upon the activity of proteases disclosed in WO 93/01800.

The invention will now be described in more detail according to the following Examples.

Examples

General Methodology

Wheat and maize diets were prepared having the following formulations:

Table 1 - Wheat diet

Ingredients	Percent
Soft Wheat	58.83
Soybean ml 48	32.49
Soy oil	4.49
Salt	0.30
Sodium Bicarbonate	0.12
DL Methionine	0.14
Limestone	1.37
Di-calcium Phosphate	1.26
Vitamins/Minerals	1.00
TOTAL	100.00

Table 2 - Maize diet

Ingredients	Percent
Maize	55.38
Soybean ml 48	37.30
Soy oil	2.96
Salt	0.30
Sodium Bicarbonate	0.16
DL Methionine	0.13
Limestone	1.22
Di-calcium Phosphate	1.55
Vitamins/Minerals	1.00
TOTAL	100.00

Animal feeds were prepared by introducing a cereal carrier containing approximately 3 mg enzyme protein/kg into the wheat diet at a concentration of 1 kg of enzyme and carrier per tonne of wheat diet. The final concentration of enzyme protein in the feed was thus approximately 3 mg per tonne. The xylanase was obtained from *Trichoderma longibrachiatum*. Broiler chicks were fed the wheat plus xylanase diet from hatching. For comparison purposes, separate flocks of chicks were fed with the wheat diet and the maize diet without the addition of xylanase. A challenge model was used, whereby

0.2 ml of a stock solution of *C. jejuni* were introduced to each chick by syringe at day 7 from hatching. The dilution level of the stock solution was varied, from 10^{-1} (high challenge) to 10^{-6} (low challenge, similar to natural conditions). The undiluted stock solution contained approximately 10^{4} CFU per 0.2 ml. The caecae of the chicks were examined for damage due to the *Campylobacter* at varying ages.

Example 1

Figures 1-6 show the effect of the diets on *Campylobacter* colonisation in 12-day old chicks. In each case two flocks, A and B, were tested to minimise the effect of environmental variance on the results. In each case it is clearly evident that a wheat plus xylanase diet is effective in reducing the level of *Campylobacter* in the caecae of the chicks in comparison with a maize diet. Additionally, at *Campylobacter* stock solution dilution levels of 10^{-3} or lower (i.e. approaching more natural conditions), the wheat plus xylanase diet becomes considerably more effective than the wheat diet alone. Thus, in Figure 1, for the wheat plus xylanase diet at a *Campylobacter* stock solution dilution of 10^{-6}, a mean score of 0.5 positive caecae was observed. The equivalent scores for diets lacking xylanase were approximately 1.5 and 2.5.

Example 2

Figures 7-9 demonstrate the effectiveness of the diets on *Campylobacter* colonisation of the small intestine and caecae of 17-day old chicks from two flocks. The effect of the wheat plus xylanase diet on reducing the *Campylobacter* colonisation of the caecae of the chicks is evident as already demonstrated in Example 1. However this reduction is even more marked as regards the small intestine. Accordingly, in Figure 7 the mean count $\log_{10}$CFU in the small intestine measured for chicks on the wheat and xylanase diet was less than 4. The equivalent counts for the diets not containing xylanase were found to be approximately 6, i.e. 100-fold higher.

Example 3

Figures 10-12 depict a comparison of the effect of different diets on the weight of 1, 5, 12, 19, 25 and 33-day old chicks. Figure 10 shows the results for the wheat-based diet, Figure 11 the results for the wheat plus xylanase-based diet and Figure 12 the results for the maize-based diet. The weight of the chicks in each case is reduced by dosing with *Campylobacter*. However, those chicks to which *Campylobacter* has been introduced gain weight more quickly on the wheat plus xylanase diet than on either of the other diets.

Example 4

Figure 13 demonstrates the effectiveness of the wheat and wheat plus xylanase diets on *Salmonella enteritidis* colonisation of the caecae of 14-day old chicks from two flocks, A and B. The methodology employed in these experiments was identical to that employed for the *Campylobacter* experiments described above, except that the undiluted stock solution of *Salmonella enteritidis* contained approximately 10^5 CFU per 0.2 ml. The effect of the wheat plus xylanase diet on reducing the *Salmonella* colonisation of the caecae of the chicks is clearly evident. Thus, in flock B, the chicks on the wheat diet were found to have a mean count $\log_{10}$CFU/ml of approximately 7. However, the chicks from flock B on the wheat plus xylanase diet were found to have a much lower $\log_{10}$CFU/ml of approximately 4 (1000-fold lower).

The above Examples clearly show a reduction in bacterial infection in the gut due to the inclusion of xylanase in the diet. Similar results have been observed when using a cellulase such as a β-glucanase. This indicates that the use provided by the present invention significantly reduces the ability of certain bacteria to colonise the caecae which in turn prevents migration of the bacteria to the small intestine. Accordingly, since it has a reduced level of infection, the growth rate of the animal is increased, leading to economic benefits. The reduction in contamination rate also has obvious benefits to human

health and the replacement of antibiotics by such diets has clear environmental benefits.